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Effects of Ractopamine HCl on *Escherichia coli* O157:H7 and *Salmonella* In Vitro and on Intestinal Populations and Fecal Shedding in Experimentally Infected Sheep and Pigs

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Abstract. The effects of the β-agonist ractopamine, approved for use in finishing swine and cattle to improve carcass quality and performance, were examined on two important foodborne pathogens, *Escherichia coli* O157:H7 and *Salmonella*. Ractopamine, administered to sheep before and after oral inoculation with *E. coli* O157:H7, increased ($P < 0.01$) fecal shedding and tended to increase ($P = 0.08$) cecal populations of the challenge strain. Pigs receiving ractopamine in the diet and then experimentally infected with *Salmonella* Typhimurium, had decreased ($P < 0.05$) fecal shedding and fewer ($P = 0.05$) liver samples positive for the challenge strain of *Salmonella*. Pure cultures of *E. coli* O157:H7 (used in the present sheep study), *E. coli* O157:H19 (isolated from pigs with postweaning diarrhea), *Salmonella* Typhimurium (used in the present pig study), and *Salmonella* Choleraesuis were incubated with varying concentrations of ractopamine to determine if ractopamine has a direct effect on bacterial growth. No differences in growth rate were observed for either strain of *E. coli* or for *Salmonella* Typhimurium when incubated with increasing concentrations of ractopamine. The growth rate for *Salmonella* Choleraesuis was increased with the addition of 2.0 μg ractopamine/ml compared with the other concentrations examined. Collectively, these results indicate that ractopamine may influence gut populations and fecal shedding of *E. coli* O157:H7 and *Salmonella*. Because ractopamine is currently approved to be fed to finishing cattle and swine immediately before slaughter, any potential for decreasing foodborne pathogens has exciting food safety implications.

The pathogenic bacteria *Escherichia coli* O157:H7 and *Salmonella* are two of the most common agents of foodborne illness in humans [1]. *E. coli* O157:H7 and *Salmonella* have been isolated from dairy and beef cattle at all stages of production [2–4], and ruminants are generally considered natural reservoirs for these bacteria [5]. Although *E. coli* O157:H7 is not considered an important foodborne pathogen in swine in the United States, *Salmonella* has received considerable attention from the swine industry, not only from a food safety standpoint, but also because *Salmonella* can cause clinical infection in swine [6].

The mechanisms of colonization and infection of these bacteria are only partially understood. Recent research has discovered a mechanism of cell-to-cell signaling used by bacteria called ''quorum sensing,'' which involves hormone-like compounds called ''autoinducers,'' that bacteria use to sense their own populations as well as the populations of other bacteria [7]. Termed ''a global regulatory mechanism for basic physiologic functions of *E. coli* O157:H7,'' this communication

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system is involved in a variety of bacterial functions, including gene expression, pathogenesis, metabolism, growth and division, and protein biosynthesis [7]. Quorum sensing has been speculated as the ''language'' that certain Gram-negative bacteria use to communicate with host cells [8]. The catecholamine hormones epinephrine and norepinephrine have been reported to be involved in a bacterial quorum-sensing system used by *E. coli* O157:H7 [8–11].

Ractopamine hydrochloride is a synthetic β-agonist that repartitions nutrients to increase lean muscle deposition, decreases the rate of adipose tissue deposition [12, 13], and improves performance, carcass leanness, and dressing percentage in finishing pigs [14, 15]. Recently approved by the United States Food and Drug Administration (USDA), ractopamine is also used in finishing beef cattle for similar performance and carcass enhancements. The catecholamine hormones norepinephrine and epinephrine are considered the physiologic counterparts to synthetic β-agonists such as ractopamine [16]. Based on the similarities of ractopamine and the catecholamines, we hypothesized that ractopamine would have ''stimulatory'' effects on *E. coli* O157:H7 similar to endogenous catecholamines. Research conducted at our laboratory using feedlot cattle naturally colonized with *E. coli* O157:H7 demonstrated that ractopamine supplementation decreased, not increased as we hypothesized, fecal shedding of *E. coli* O157:H7 [17]. To further investigate these findings, a series of experiments were conducted to determine (1) the effects of ractopamine on gut populations and subsequent fecal shedding of *E. coli* O157:H7 and *Salmonella* in experimentally infected sheep and pigs and (2) the effect of ractopamine on *E. coli* O157:H7 and *Salmonella* in vitro.

Materials and Methods

Bacterial cultures. *E. coli* O157:H7 strain 933 (ATCC 43895), associated with raw hamburger implicated in a case of human hemorrhagic colitis, was obtained from the American Type Culture Collection (Manassas, VA). *E. coli* O157:H19 (F18 fimbriae) was cultured from pigs with postweaning diarrhea and identification confirmed by the Gastroenteric Disease Center, Penn State University. *Salmonella* serovar Typhimurium was obtained from the National Veterinary Services Laboratory (Ames, IA), and serovar Choleraesuis was provided by Paula Fedorka-Cray, Athens, GA. Strains used in the animal experiments and not naturally resistant to novobiocin and naladixic acid were made resistant to 25 and 20 μg/ml novobiocin and naladixic acid, respectively. Bacterial strains were cultured in anoxic tryptic soy broth (TSB) medium at 37°C, and bacteria that were stable through three successive overnight transfers were used in the following experiments.

Animal experiments. Sheep were used to determine the effects of ractopamine supplementation on gut populations of *E. coli* O157:H7 in ruminants. Sixteen crossbred market lambs were housed outdoors in covered pens (4 head/pen) and gradually adjusted to an 80:20 concentrate-to-hay diet during a 10-day period. After diet acclimation, lambs were randomly assigned to treatment (control [empty gelatin capsule] or ractopamine [Paylean 9; Elanco Animal Health, Indianapolis, IN; 0.95 mg ractopamine/kg body weight]) administered daily for 28 days by way of oral bolus. On day 21, sheep were weighed and moved to individual indoor pens, and fecal grab samples were collected from each animal. Feces were vortexed in phosphate-buffered saline (PBS) and plated on MacConkey's agar (MAC_{nn}) supplemented with novobiocin (25 μg/ml) and naladixic acid (20 μg/ml) to screen for wild-type *E. coli* capable of growth on MAC_{nn}. On day 23, lambs were inoculated by way of oral gavage with *E. coli* O157:H7 (18×10^8 in 10 ml TSB). Individual fecal grab samples were collected daily for 4 days, and 1 g feces was serially diluted in PBS and plated on MAC_{nn} for quantitative enumeration of the inoculated strain. On day 29, all lambs were humanely killed (Euthasol; Delmarva Laboratories, Midlothian, VA) and necropsied. Tissue and luminal contents were aseptically collected from the rumen, cecum, ileum, colon, and rectum. One gram of contents from each gut segment was serially diluted in PBS, plated on MAC_{nn}, and incubated as previously described. Tissue samples were enriched in gram negative Hajna broth (37°C for 24 hours) before plating on MAC_{nn} and incubating as previously described. Feed and water were available throughout the experimental period for ad libitum consumption. Lambs were weighed on days 7 and 21, and ractopamine dosages were adjusted accordingly.

For the swine study, 16 crossbred barrows and gilts (average body weight 33 kg) were purchased from the Texas Department of Criminal Justice and transported to our facilities in College Station, TX. On arrival, pigs were weighed, ear-tagged, and randomly assigned to pens (8 pigs/pen) and to treatment (control [grower ration only] or ractopamine [grower ration supplemented with 18 g/ton Paylean 9]). Sex was equally represented in each treatment. Feed and water were provided for ad libitum consumption throughout the experimental period. Fourteen days after initiation of treatments, all pigs were individually penned and fed their respective diets for an additional 24 days (38 days total on treatment). After individual penning, fecal grab samples were collected and plated on brilliant green agar supplemented with novobiocin (25 μg/ml) and naladixic acid (20 μg/ml) (BGA_{nn}) to screen for the presence of wild-type *Salmonella* before inoculation with the experimental strain. On day 34, all pigs were inoculated with *Salmonella* Typhimurium (4.5×10^7 in 5 ml TSB) by way of oral gavage. Fecal grab samples were collected daily for 4 days from individual pigs for quantification of the inoculated strain. One gram of feces was serially diluted in PBS and plated on BGA_{nn}, incubated (37°C for 24 hours), and colonies expressing typical *Salmonella* morphology were counted. Four days postchallenge, pigs were sedated with an intramuscular injection of cocktail containing Ketaset, Telazol (Ft. Dodge Laboratories, Kansas City, KS) and Xylazine (Phoenix Scientific, St. Joseph, MO) and killed humanely with Euthasol. Tissue and luminal contents were aseptically collected from the ileum, cecum, colon, and rectum. Tissue samples were also collected from the ileocecal lymph nodes, spleen, and liver. All tissue samples were enriched in tetrathionate broth (37°C for 24 hours), plated on BGA_{nn}, and incubated (37°C for 24 hours) for qualitative determination of the challenge strain.

The Animal Care and Use Committee of the Food and Feed Safety Research Laboratory, USDA preapproved the care, use, and handling of all experimental animals. Unless otherwise noted, all media and agar were from Difco Laboratories (Detroit, MI) and reagents from Sigma Chemical (St. Louis, MO).

In vitro effects of ractopamine. Pure cultures of the *E. coli* strains and *Salmonella* serovars described previously were individually added (0.5 ml) to 9 ml autoclaved TSB. Ractopamine HCl dissolved in anoxic distilled water was added to achieve the desired final concentrations of 0, 0.5, 1, 2, 4, and 8 μg ractopamine/ml in 10 ml total volume. The tubes were sealed, vortexed, and incubated at 37°C throughout the experiment. Growth rates were determined by way of measurement of optical density (OD) at 600 nm using a Spectronic 20D spectrophotometer (Rochester, NY) by taking cell-density readings every 30 minutes until an OD of 0.6 was reached. The experiment was replicated three times on separate days. The maximal specific growth rate (μ) was calculated using the formula: $\mu = (\ln OD^2 - \ln OD^1)/\Delta T$, where ΔT = time change during logarithmic growth phase for each culture.

Statistical analyses. Data were analyzed using SAS version 8.02 (SAS, Cary, NC). Data from multiple repetitions of the in vitro experiments were pooled before analysis. Bacterial growth rates, gut concentrations of inoculated pathogens, and body weights were subjected to analysis of variance appropriate for a completely randomized design. Daily fecal shedding data was analyzed using the Proc Mixed procedure for repeated measures with treatment, day, and treatment × day interaction included in the model. The incidence of positive tissue samples was subjected to χ^2 analysis using the PROC FREQ procedure. Differences among means were considered significant at a 5% level of significance and means separated when appropriate using Duncan's Multiple Range Test.

Results

Animal experiments. For the sheep study, culture of individual fecal samples from all lambs demonstrated there no wild-type *E. coli* was capable of growth on MAC_{nn} before experimental infection. A treatment ($P = 0.0003$) effect and treatment × day ($P = 0.01$) interaction, but no day effect ($P > 0.10$), were observed for daily fecal shedding of the challenge strain of *E. coli* O157:H7 (Fig. 1). Ractopamine-treated lambs shed more *E. coli* O157:H7 in feces on each day of the 4-day collection period (particularly on day 1 postchallenge) than control lambs. When examined across day, lambs shed 3.79 and 5.04 colony-forming units (CFU)/g feces (log_{10}) for the control and ractopamine treatments, respectively. Populations of *E. coli* O157:H7 in the luminal contents of the rumen, ileum, colon, and rectum were not different ($P > 0.10$) among treatments; however, ractopamine-treated animals tended ($P = 0.08$) to have greater populations of the challenge strain in cecal contents compared with control lambs (Table 1). The number of tissue samples from the rumen, ileum, cecum, colon, and rectum positive for *E. coli* O157:H7 were not different due to treatment (Table 1).

Body weights were similar ($P > 0.10$) at the initiation of the experimental period. Lambs in both treatments lost weight during the course of the experiment, with a greater ($P = 0.009$) loss observed in control lambs compared with ractopamine-treated lambs. Feed intakes were not recorded, but no obvious visual differences were observed.

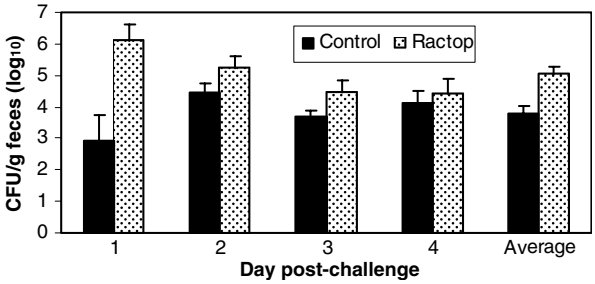

Fig. 1. Fecal shedding (CFU/g feces log_{10}) of the challenge strain of *E. coli* O157:H7 in lambs dosed daily with ractopamine (0.95 mg/kg body weight) for 28 days and inoculated on day 23.

Table 1. Daily fecal shedding and luminal content populations of *E. coli* O157:H7 (CFU/g log_{10}), *E coli* O157:H7 positive tissue samples and body weight change in lambs experimentally infected with *E. coli* O157:H7 and dosed daily with ractopamine (0.95 mg/kg/body weight/d for 28 days)

	Treatment			
	Control	Ractopamine	SEM[a]	*P* value
Daily shedding[b]	3.79[c]	5.04[d]	0.23	0.0003
Luminal contents				
Rumen	1.16	1.21	0.17	0.82
Ileum	1.81	3.13	0.63	0.16
Cecum	2.37	3.46	0.41	0.08
Colon	2.61	3.42	0.43	0.20
Rectum	2.85	2.71	0.52	0.85
Tissue enrichments				
Rumen	4/8	6/8		0.30
Ileum	5/8	5/8		1.0
Cecum	8/8	8/8		1.0
Colon	7/8	8/8		0.30
Rectum	5/8	6/8		0.59
Body weight (kg)				
Initial	46.6	46.3	1.34	0.89
Final	42/1	44.4	1.41	0.28
Change	−4.5[c]	−1.9[d]	0.58	0.009

[a]Standard error of the mean.
[b]No treatment by day interaction ($P > 0.20$); therefore, data were pooled and averaged across days.
[c,d]Values within row without a common superscript differ ($P < 0.05$).

For the pig study, fecal samples collected from all pigs and cultured before inoculation with the experimental *Salmonella* Typhimurium strain were negative for wild-type *Salmonella* capable of growth on BGA_{nn}. Fecal shedding data of the challenge strain of *Salmonella* is listed by day (Fig. 2), although no treatment x day interaction was observed ($P > 0.10$). Populations of *Salmonella* in the feces decreased ($P < 0.01$) with time,

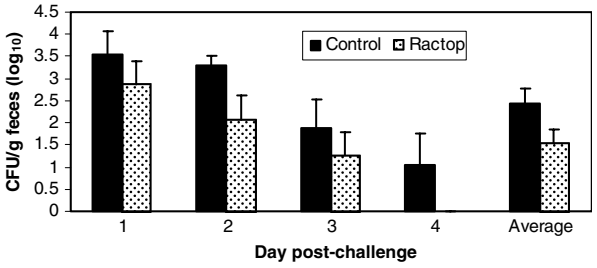

Fig. 2. Fecal shedding (CFU/g feces $\log_{10}$) of the challenge strain of *Salmonella* Typhimurium in feeder pigs fed diets with and without ractopamine (18 g/ton feed) for 38 days and inoculated on day 34.

and low levels of *Salmonella* were detectable only in the control animals on day 4. *Salmonella* shedding was higher in the control animals each day postchallenge and when examined across days ($P < 0.01$; Fig. 2).

Luminal content populations and the number of tissue samples positive for the challenge strain of *Salmonella* are listed in Table 2. Populations of *Salmonella* in the luminal contents collected from the ileum, cecum, colon, and rectum were not significantly different. A numeric decrease ($P = 0.10$) in *Salmonella* population was observed in the colon contents in ractopamine-fed pigs compared with controls. The number of *Salmonella*-positive tissue samples was largely unaffected by ractopamine supplementation, and no treatment differences ($P > 0.10$) were observed in the number of positive tissues from the cecum, ileum, colon, rectum, or spleen. The number of *Salmonella*-positive liver tissue samples was higher ($P = 0.05$) in the control animals, whereas the number of positive ileocecal lymph nodes was numerically higher ($P = 0.13$) in the control compared with the ractopamine-treated animals.

Body weights were similar ($P > 0.10$) at the initiation of the experimental period. Pigs fed ractopamine tended ($P = 0.13$) to gain more weight during the 38-day experimental period compared with controls (Table 2). Feed intakes were not different ($P > 0.10$) among treatments (data not shown).

In vitro experiment. We selected multiple concentrations of ractopamine for testing based on our approximations of gut ractopamine concentrations in feedlot steers and finishing pigs fed ractopamine according to the manufacturer's recommendations. Growth rates for *E. coli* O157:H7 and O157:H19 were not affected ($P > 0.10$) by exposure to increasing concentrations of ractopamine (Table 3). Likewise, growth rates of *Salmonella* Typhimurium were not affected ($P > 0.10$) by ractopamine exposure at any of the concentrations examined. However, exposure to 2.0 μg/ml ractopamine increased ($P = 0.03$) the growth rate of *Salmonella* Choleraesuis compared with the other ractopamine concentrations examined (Table 3).

Table 2. Daily fecal shedding and luminal content populations of *Salmonella* (CFU/g $\log_{10}$), *Salmonella-positive* tissue samples, and body weight change in feeder pigs experimentally infected with *Salmonella* and fed a grower ration with and without ractopamine (18 g/ton)

	Treatment			
	Control	Ractopamine	SEM[a]	*P* value
Daily shedding[b]	2.44[d]	1.55[e]	0.25	0.01
Luminal contents				
Ileum	1.87	1.30	0.32	0.22
Cecum	2.51	2.04	0.40	0.42
Colon	2.22	1.33	0.45	0.18
Rectum	1.19	0.50	0.51	0.35
Tissue enrichments				
Cecum	8/8	8/8		1.0
Ileum	7/8	8/8		0.30
Colon	7/8	6/8		0.52
Rectum	3/8	4/8		0.61
LN[c]	5/8	2/8		0.13
Spleen	1/8	1/8		1.0
Liver	3/8[d]	0/8[e]		0.05
Body weight (kg)				
Initial	33.5	32.9	1.12	0.75
Final	66.8	68.1	1.38	0.51
Change	33.3	35.2	0.79	0.13

[a]Standard error of the mean.
[b]No treatment by day interaction ($p > 0.20$); therefore, data were pooled and averaged across days.
[c]Ileocecal lymph nodes.
[d,e]Values within row without a common superscript differ ($p < 0.05$).

Discussion

Although the effects of ractopamine on animal performance and carcass quality have been well documented, to our knowledge we are the first to report the effects of this compound on fecal shedding and gut populations of foodborne pathogenic bacteria. Research in vitro reported the "stimulatory" effects of the catecholamines on *E. coli* O157:H7 [8–10] and led us to hypothesize that ractopamine may act similarly. Initial research conducted in our laboratory examined beef cattle "naturally" shedding *E. coli* O157:H7 in the feces and individually dosed with ractopamine. Within 1 week of initiating ractopamine treatments, we saw a decrease in the number of animals shedding *E. coli* O157:H7, which continued throughout the 4-week experimental period [17]. Examination of plasma catecholamines failed to explain the observed treatment differences and led to a continuation of this research reported here.

Sheep, like cattle, are naturally colonized with *E. coli* O157:H7; have been documented with popula-

Table 3. Maximal specific growth rates (h^{-1}) of two strains *E. coli* O157 and two serovars of *Salmonella* exposed to multiple concentrations of ractopamine in pure culture

	Ractopamine (μg/ml)							
Bacteria	0	0.5	1	2	4	8	SEM[a]	$P > F$
E. coli								
O157:H7	0.815	0.886	0.866	0.812	0.904	0.857	0.14	0.99
O157:H19	0.842	0.818	0.852	0.916	0.85	0.87	0.07	0.95
Salmonella								
Typhimurium	0.815	0.835	0.924	0.888	0.863	0.845	0.08	0.94
Choleraesuis	0.694^{c}	0.805^{c}	0.773^{c}	1.17^{b}	$0.983^{b,c}$	0.803^{c}	0.09	0.03

[a]Standard error of the mean.
[b,c]Row means with different superscripts differ ($P < 0.05$).

tions similar to those found in cattle; and are often used as an economic experimental ruminant model for *E. coli* O157:H7 colonization [18–20]. To examine the effect of ractopamine supplementation on susceptibility to colonization and gut populations, we experimentally infected lambs with *E. coli* O157:H7. Contrary to our cattle research [17], but consistent with our initial hypothesis and with in vitro research involving catecholamines [8–10], ractopamine-treated animals shed more E. coli O157:H7 in the feces and tended to have greater populations in the cecal contents.

Caution should be used, however, to avoid overinterpretation of these results. Fecal shedding data from day 1 postinoculation is inconsistent with the remaining daily fecal shedding data. Ractopamine-treated lambs shed higher levels of *E. coli* O157:H7 on day 1 compared with control animals; however, by day 2 and throughout the remainder of the experiment, the differences among treatments were slight. This suggests that ractopamine treatment before inoculation either enhanced colonization by the inoculated strain and subsequent fecal shedding or increased the transit time of the inoculated strain through the gastrointestinal tract. However, *E. coli* O157:H7 shedding increased sharply in controls on day 2 and remained consistent and comparable with the ractopamine-treated lambs on days 3 and 4, suggesting that the day 1 data for control lambs may not have accurately reflected the effects of ractopamine supplementation at this time point. If fecal shedding data for day 1 is eliminated from the statistical analysis, a significant treatment effect is still observed ($P = 0.04$), although shedding levels are within the same log unit (4.08 and 4.69 $\log_{10}$ CFU/g feces for control and ractopamine treatments, respectively). Because lambs remained on their respective treatments throughout the entire experimental period, we would expect that the differences on day 1, if in fact a result of treatment, would have been observed on subsequent days. Although differences were observed in *E. coli* O157:H7 populations in the cecal contents in ractopamine-treated lambs, this did not correspond with an increase in fecal shedding on day 4. The mucosa of the rectal–anal junction has been suggested by some investigators as the primary site of *E. coli* O157:H7 colonization in cattle [21]; however, no differences in rectal content populations or in the number of positive rectal tissue samples were observed in the current research.

Conflicting results of this research and research in ruminants reported by the investigators previously [17] may be related to species differences (cattle vs. sheep) or in bacterial populations as a result of experimental procedures (naturally vs experimentally infected). Perhaps greater or ecologically established populations of *E. coli* O157:H7 are required before ractopamine is able to elicit an effect. This might explain the differences in this study using experimentally inoculated sheep versus the naturally infected cattle study [17], in which enrichment and immunomagnetic separation were required for qualitative assessment of fecal shedding. However, if ractopamine has a direct effect on *E. coli* O157:H7 within the gut, we would expect to observe a growth response in pure culture studies. In vitro examination of various ractopamine concentrations on the same strain of *E. coli* O157:H7 used in the sheep study failed to elicit any changes in growth rate.

Because the first regulatory approval for ractopamine was in finishing swine, we believed that research examining its effects on *Salmonella*, a Gram-negative bacterium with important food safety and pig health implications, was warranted. Conversely to what we observed in the sheep experiment with *E. coli* O157:H7, ractopamine decreased fecal shedding and produced slight decreases in gut populations of the inoculated *Salmonella* strain in pigs. In both treatment groups, fecal shedding decreased steadily during the 4-day experimental period, indicating the competitive fitness of an

established bacterial microflora. However, although fecal shedding decreased rapidly by day 4, *Salmonella* was detected in the luminal contents from all of the gut segments at necropsy, indicative of this bacterium's ability to colonize and compete in a mature digestive system. Interestingly, colonization of the ileocecal lymph nodes tended to be different, and the liver, but not the spleen, showed a treatment effect, with fewer *Salmonella*-positive samples isolated in the ractopamine-treated animals. This suggests that ractopamine may have the desirable effect of decreasing tissue invasiveness of *Salmonella*.

As mentioned previously, if ractopamine has a direct effect on the bacteria, then we expected to see an effect on growth rates in pure culture. However, when examined in pure culture with increasing concentrations of ractopamine, growth rates of *Salmonella* Typhimurium (same strain as used in the pig study) were not different. Surprisingly, growth rates of the *Salmonella* serovar Choleraesuis were increased when exposed to 2.0 μg/ml ractopamine in pure culture. The physiologic reasons underlying these serovar differences are unknown.

The differences in bacterial shedding and gut populations reported here, although intriguing not only from a food safety but also a pig health standpoint, are difficult to explain. Possibly, slight changes in gastrointestinal function or microbial ecology because of ractopamine treatment were significant enough to influence bacterial populations. If a result of a direct effect of ractopamine on the bacteria, then we would also expect to see an effect on these same bacteria in pure culture, which we did not observe. However, the use of pure-culture experiments does not incorporate the complexity of a mixed-microbial system, which could certainly influence the results. Additionally, limitations of the current methodology used in the animal experiments must be considered when interpreting these results. Sheep were used as an inexpensive cattle model. However, ractopamine has not been approved for use in sheep, and the effects of this compound may be significantly different from those observed in cattle. Because of handling and disposal considerations, we used feeder pigs weighing <70 kg. Ractopamine is intended to be fed to market-weight animals (approximately 100 kg) immediately before slaughter; obviously, age and maturity of the animals could influence the results. The use of a limited number of experimentally infected animals, although useful for initial experimentation and from a cost standpoint, does not necessarily reflect ''real-world'' conditions. Expanded studies incorporating knowledge learned from these experiments and examining ''naturally infected'' or ''colonized'' animals in modern production settings are the logical next step to continue this novel research.

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